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Short Communication

Characterization of ultrasound-induced pulmonary capillary hemorrhage in rats

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ABSTRACT

Routine pulmonary ultrasound for diagnosis of disease or injury relies on interpretation of image features, such as comet-tail artifacts, which can also be indicative of the poorly understood phenomenon of ultrasound-induced pulmonary capillary hemorrhage (PCH). Evans blue extraction and bronchoalveolar lavage (BAL) were evaluated for assessment of PCH induced by ultrasound scanning. Rats anesthetized with ketamine with or without xylazine received sham or scanning for 5 min with a 7.6 MHz linear array. Evans blue extraction and BAL albumin measurements failed to demonstrate significant increases for scanning, even though the induction of comet-tail artifacts was significant. BAL cell counts had an insignificant increase relative to shams at a near-threshold mechanical index (MI) of 0.52 ($P = 0.07$), but a highly significant increase at MI = 0.9 ($P = 0.001$). The possibility of xylazine-induced elevated albumin was tested, but no significant decrease was found for sham or scanned rats with ketamine-only anesthesia. Interestingly, without xylazine, the widths of comet-tail artifacts in the ultrasound images were significantly smaller ($P = 0.001$) and cell counts in BAL fluid also were reduced ($P = 0.014$). The BAL cell-count method provides a valuable additional means of PCH quantification.

Introduction

Diagnostic ultrasound imaging of the lung can induce pulmonary capillary hemorrhage (PCH). The induction of PCH by pulsed ultrasound was discovered more than 20 yrs ago by Child et al. (1990). Research has indicated that the phenomenon occurs in mice, rats and pigs and may be characterized by a threshold for a specific situation (American Institute of Ultrasound in Medicine, 2000; Church et al. 2008). Initially, the lung was expected to receive mostly incidental exposure, such as during echocardiography, and therefore the risk of patient injury was thought to be low. However, the use of trans-thoracic diagnostic ultrasound for direct lung examination has grown rapidly in recent years (Volpicelli, 2013). Various features in the images, such as comet-tail artifacts (CTAs), are interpreted to diagnose interstitial syndrome, pulmonary edema and pulmonary effusion, among others. More research is needed to define the possible risks of PCH for patients and to provide suitable safety guidance.

In a previous study, a diagnostic ultrasound machine (HDI 5000, Philips Healthcare, Andover, MA, USA) with 7.6 MHz (CL15-7) linear array was used to image the right lung of anesthetized rats in a warmed water bath (Miller 2012). The image immediately displayed growing comet-tail artifacts (CTAs) for a mechanical index (MI) of 0.9. Upon examination of the lung, a hemorrhage region corresponded to the region of CTAs in the scan plane. PCH was observed for several groups of rats scanned at a range of MI settings, and a threshold was indicated for an MI of about 0.44. This result indicates a greater sensitivity to direct pulmonary ultrasound than was expected from earlier findings.

Previously, the PCH was observed by ultrasound imaging and by stereomicroscopy of the excised lungs (Miller 2012). A quantitative measure of PCH volume would also be a valuable parameter. Histological assessment of PCH volumes is difficult and somewhat uncertain due to distortion during fixation and processing. In this study, two alternative methods were tried. First, Evans blue IV injection before scanning, with subsequent extraction of the dye from lung tissue samples, was tested as a PCH characterization method. This method has been used to characterize various types of edema (Green et al. 1988), including pulmonary edema as a measure of assessing permeability injury (Kelher et al. 2009). Second, bronchoalveolar lavage (BAL), which is

Abbreviations: BAL, bronchoalveolar lavage; MI, mechanical index; PCH, pulmonary capillary hemorrhage; CTA, comet tail artifact; MWRS, Mann–Whitney rank sum.

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useful in research on lung contusion (Raghavendran et. al. 2005; 2008), was used to characterize the ultrasonic PCH by measurement of albumin and cell counts in BAL fluid.

Materials and methods

All *in vivo* animal procedures were conducted with the approval and guidance of the University Committee on Use and Care of Animals (UCUCA). 41 female rats (CD IGS strain, Charles River, Wilmington, MA, USA) 8–10 weeks of age and weighing an average of 223 g (st. dev. 13 g) were used for this study, with three lost from the study due to technical problems. The general methods were described previously (Miller 2012). Anesthesia with ketamine (91 mg kg^{-1}) and xylazine (9 mg kg^{-1}) was used for 33 rats (2 lost), and ketamine alone (100 mg kg^{-1}) was used for 8 rats (1 lost). The purpose of the omission of the xylazine for anesthesia in some rats was to evaluate the possibility of xylazine-induced elevation of permeability. Xylazine has been reported to induce pulmonary edema, although with higher doses of 21-42 mg/kg (Amouzadeh et al. 1991, 1993). The right thorax of all rats was shaved and depilated for ultrasound transmission. The rats were mounted in a 38 °C degassed water bath for ultrasound exposures of the right lung. This exposure method provides reproducible ultrasound coupling and exposure, and maintains the body temperature of the rats. Ten minutes after scanning or sham scanning, the rats were sacrificed by exsanguination under anesthesia for evaluation of the lungs.

A Phillips HDI 5000 (Philips Healthcare, Andover, MA, USA) diagnostic ultrasound machine with CL15-7 linear array was used, as described previously (Miller 2012). This probe was set up in the water bath to scan the right cranial or middle lobe in B mode for 5 min with 2 cm image depth, 1 cm focal depth, and 39 frames per second. The probe was partially in contact with the skin, and the pleural surface was at a depth of about 5–6 mm. The center frequency was 7.6 MHz with a pulse repetition frequency of 10 kHz. The MI level was set by the on-screen readout to 0.52, which was just above the PCH threshold, or to the maximum 0.9. These settings were previously estimated to yield in situ peak rarefactional pressure amplitudes of about 1.2 or 1.9 MPa, respectively. The size of the region with PCH was estimated from the width of the CTAs in the lung image, and by measurement of the hemorrhage area on excised lungs. The PCH also was characterized by two new methods: Evans blue extraction and bronchoalveolar lavage (BAL). Tissue samples from the scanned region of the lung after BAL also were fixed in neutral buffered formalin and processed for histology by the University of Michigan Comprehensive Cancer Center, Research Histology and Immunoperoxidase Laboratory, to detect the retention of cells in the lung after BAL.

The Evans blue evaluation was modeled after published methods (Green et al. 1988; Kelher et al. 2009). Evans blue at 20 mg/ml in saline was injected at 30 mg/kg via tail vein at anesthesia. Evans blue has a high affinity for albumin, and therefore is an indicator for capillary permeability. At sacrifice, blood was obtained for a plasma sample, and the lung circulation was cleared by 20 ml phosphate buffered saline perfusion into the right ventricle. The trachea was occluded and the lung was removed intact for photography. The length and width of the hemorrhage region were measured on the lungs. Right lung lobes (cranial or middle lobe with PCH for scans, or both cranial and middle lobe for shams) were removed and placed in 9 times the volume of the lung sample (by weight) of formamide (F-5786, Sigma-Aldrich, St. Louis, MO, USA) for extraction. The lung samples were placed in formamide, minced, subjected to a brief vacuum to reduce the amount of gas remaining in the lungs, and then incubated at 60 °C overnight. After centrifugation at 3200 ×*g* for 30 min the supernatant fluid was measured using a spectrophotometer for Evans blue absorbance at 620 nm. For measuring the optical densities, the extracted samples were measured undiluted after centrifugation, while the plasma samples were diluted to 4 μl/ml with formamide.

The BAL evaluation followed the methods of Raghavendran et al. (2005, 2008). The chest was opened and the lung circulation was cleared as described above. The right lung was isolated and instilled with 5 ml of phosphate buffered saline (PBS pH 7.4, Gibco, Life Technologies Corp., Grand Island NY, USA) twice with collection of the BAL fluid each time. The BAL procedure precluded size measurement on the lungs. For the higher MI exposures, the fluid was typically pink due to the presence of erythrocytes. The second lavage sample gave reduced results, judging by the pink color of the samples, and only the first sample was analyzed. The samples were centrifuged to separate the cells, which were counted using a cell counter (Multisizer 3, Beckman Coulter, Fullerton, CA). The supernatant was assayed for albumin concentration using an enzyme-linked immunoassay kit (GenWay Biotech Inc., San Diego, CA).

For the Evans blue testing, the groups with ketamine and xylazine anesthesia included 4 shams, 3 rats scanned at MI = 0.52 and 4 rats scanned at MI = 0.9. For the BAL testing with ketamine and xylazine anesthesia, the groups included 7 shams, 4 scanned at MI = 0.52 and 6 scanned at MI = 0.9. In addition, groups of 4 rats were anesthetized with ketamine alone for sham and MI = 0.9 scanning, to test the influence of xylazine on the results. Statistical analyses between groups were performed by the *t*-test or the Mann–Whitney Rank Sum (MWRS) test using SigmaPlot for Windows V. 11.0 (Systat Software Inc., San Jose, CA, USA), with statistical significance assumed at $P < 0.05$. In addition, a two way ANOVA (Holm–Sidek test) was used to gauge the relative importance and interaction of xylazine and scanning in the BAL cell count results.

Results

The results of the Evans blue method are presented in Table 1. One animal had a negative outcome (no visible PCH) for scanning at MI = 0.52. The optical densities indicated a low level of albumin in the lung by comparison to the diluted plasma samples (Table 1). The results for the scanned groups were not significantly different from sham results. The lengths of the CTAs in the images and the measured hemorrhage areas on the lungs (Table 1) were both significantly increased relative to the zero values in shams for the MI = 0.9 scans, but not for MI = 0.52 with only two of the three outcomes positive for PCH. Therefore, the Evans blue extraction method was not sufficiently sensitive to pick up the ultrasound-induced PCH.

The results of the BAL cell-count method are shown in Fig. 1. For ketamine plus xylazine anesthesia, the MI = 0.52 increase was insignificant relative to shams ($P = 0.07$ MWRS-test), but the MI = 0.9 increase was highly significant ($P = 0.001$ MWRS-test). Residual erythrocytes were often noted in the lung after lavage, as shown in Fig. 2a. Although this residual feature was initially thought to be due to capillary hemorrhage into the lung interstitium, histological examination of the lavaged lobe revealed that the red cells were primarily in the alveolar space, see Fig. 2b. This suggests that the cell counts did not completely reflect the true amount of PCH, due to limited retrieval of the cells from alveolar spaces. The width of the lung surface image with comets was 5.8 ± 4.7 mm (not significant with 3 positive and one negative result) for MI = 0.52, and 10.9 ± 1.7 mm ($P < 0.01$, MWRS-test)

Table 1
Results for means and standard deviations for the Evans blue test method.

Group MI	Weight g	Sample OD	Plasma OD	Area mm^2	Length mm
Sham	0.27 ± 0.07	0.26 ± 0.14	0.17 ± 0.03	0.0	0.0
0.52	0.19 ± 0.03	0.47 ± 0.10	0.18 ± 0.01	1.4 ± 1.9	1.4 ± 1.5
0.9	0.18 ± 0.01	0.49 ± 0.23	0.17 ± 0.01	18 ± 7*	10.1 ± 3.1*

The PCH areas from lung surface measurements and lengths from the ultrasound images were found to be statistically significantly different (*) from the corresponding sham for MI = 0.9. Data presented as the means with standard deviations: MI, Mechanical Index of exposure; OD, optical density.